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Distribution of Urethane and Its Binding to DNA, RNA, and Protein in SENCAR and BALB/c Mice Following Oral and Dermal Administration

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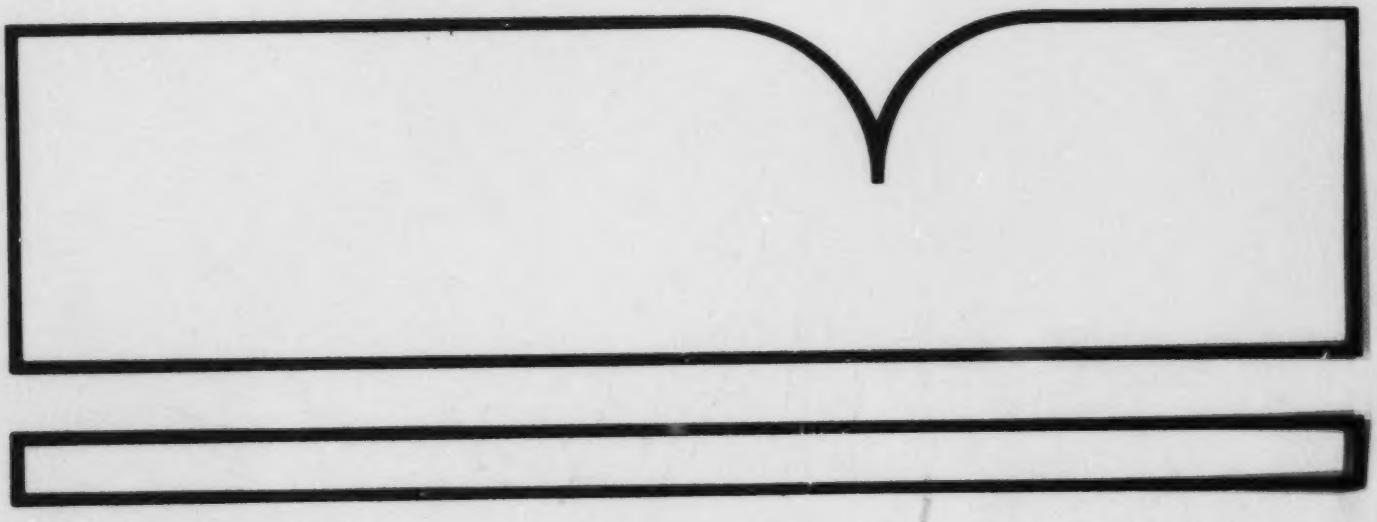

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16. ABSTRACT

In the initiation-promotion (I/P) assay for mouse skin tumorigenesis the initiating activity of various compounds is dependent upon both the route of administration and strain of mouse tested. Urethane produces 3-fold more skin papillomas per mouse when administered orally than dermally in Sencar mice. To examine the biochemical basis for route and strain differences in the I/P assay, the binding of ^{14}C-urethane to DNA, RNA, and protein in mice susceptible (Sencar) and relatively resistant (Balb/c) to tumorigenesis by this protocol was determined. Binding of ^{14}-C-urethane (0.062 mg/g body weight, 50 uCi/20 g body weight) to DNA, RNA and protein 6 hours after oral administration varied with tissue (liver > stomach > skin = lung) but not with strain. Binding to DNA in skin, lung and stomach, RNA in stomach and protein in stomach and liver after 48 hours was significantly higher in Sencar than in Balb/c. Dermal application of ^{14}C-urethane resulted in several fold higher binding to liver DNA of Sencar mice than Balb/c mice. Forty-eight hours after dermal application, significantly higher levels of ^{14}Curethane remained bound to skin DNA, RNA and protein Balb/c mice, although all values were lower than at 6 hours after treatment. These results suggest that levels of urethane bound to DNA after oral and dermal exposure are comparable and that the difference in tumor induction after initiation by these two protocols cannot be explained by differences in binding to any macromolecule.

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DISTRIBUTION OF URETHANE AND ITS BINDING TO DNA, RNA, AND PROTEIN IN SENCAR AND BALB/c MICE FOLLOWING ORAL AND DERMAL ADMINISTRATION

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Urethane produces threefold more skin papillomas when administered orally than dermally in SENCAR mice, a strain susceptible to tumorigenesis. To better understand the relation of distribution to the initiation stage, [^{14}C]urethane (0.10 mg/kg, 2.5 μCi/25 g) was administered orally and dermally to male SENCAR and BALB/c mice. Absorption of urethane was greater in the first hour in SENCAR mice by both routes, as indicated by more label in the liver, lung, and stomach than found in these tissues in BALB/c mice. These differences were not observed at later time periods after oral administration. Following dermal application, higher levels were maintained in the liver, lungs, and stomach through 48 h in the SENCAR mice when compared to BALB/c mice. Binding of [^{14}C]urethane (0.062 mg/g body weight, 20 μCi/20 g body weight) to DNA, RNA, and protein 6 h after oral administration varied with tissue (liver > stomach > skin = lung) but did not differ with strain. Binding to DNA in skin, lung, and stomach, RNA in stomach, and protein in stomach and liver after 48 h were significantly higher in SENCAR mice than in BALB/c mice. Dermal application of [^{14}C]urethane resulted in severalfold higher binding to liver DNA of SENCAR mice than BALB/c mice, but DNA binding was comparable in other tissues after 6 h. At 48 h after dermal application, significantly higher levels of [^{14}C]urethane remained bound to skin DNA, RNA, and protein in BALB/c mice, although all values were lower than at 6 h after treatment. Differences in the distribution and binding of urethane probably do not account for the discrepancies in tumor sensitivity. Liver DNA hydrolysates were examined after 48 h. Thin-layer chromatography showed little incorporation of the ^{14}C into the normal deoxyribonucleotide or deoxyribonucleoside bases, and no modified bases were apparent. Radioactivity was present in the fraction that remained at the origin and was consistant with a dinucleotide fragment resistant to phosphodiesterase cleavage, such as a phosphotriester.

The authors are grateful for the technical assistance of Patrice Weaver. This study was supported in part by a grant from the Indiana Elks through the Purdue Cancer Center, National Research Service Award 5T32ES07039, and by the United States Environmental Protection Agency under assistance agreement number CR809744010. It has not been subjected to the Agency's peer and administrative review and, therefore, does not necessarily reflect the view of the agency and no official endorsement should be inferred.

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INTRODUCTION

One of the most promising methods for determining the tumorigenicity of a chemical is the initiation-promotion assay in SENCAR mice, which have been bred for their sensitivity (DiGiovanni et al., 1980). After application of the test compound followed by a 20-wk promotion schedule, papilloma formation on the skin of the mice is quantitated. As more compounds are tested, however, there appear to be differences in the tumor incidence in accordance with changes in the route of administration and strain (Slaga et al., 1982).

Urethane (ethyl carbamate) has been shown to cause tumors in a number of tissues including lung (Nettleship et al., 1943; Yamamoto et al., 1971), liver (Heston et al., 1960, Klein, 1966), stomach (Berenblum and Haran-Ghera, 1957), and skin (Salaman and Roe, 1953, Pound and Bell, 1962). Bull et al. (1981) determined the mouse skin response to initiating doses of carcinogens following the single administration of initiators by different routes. When urethane was administered orally, ip, sc, and dermally (0.5 mg/g) to SENCAR mice followed by a 20-wk promotion schedule, the response to urethane given orally was threefold that of the dermal route. Benzo[a]pyrene on the contrary induced 10-fold more papillomas at doses above 0.1 mg/g by the dermal route versus the oral route. Dahl et al. (1978) also demonstrated that intraperitoneal doses of carbamates (0.01–0.065 mg/g body weight) produced 2–3 times more skin papillomas than did dermal doses in CD-1 mice.

Papilloma formation in the initiation-promotion assay in mouse skin also varies with strain. Hennings et al. (1981) demonstrated an increased sensitivity to tumor induction by dimethylbenzo[a]anthracene in the SENCAR mouse regardless of the route of administration, age, or sex when compared to the BALB/c strain. Robinson et al. (1984) found that the intraperitoneal administration of urethane (300 mg/kg 3 times weekly for 20 wk) caused an average of 0.80 tumors/SENCAR mouse but only 0.08 tumors/animal for BALB/c mice. Similar results have been shown for other tumorigens, including benzo[a]pyrene (Bull et al., 1983) and acrylamide (Bull et al., 1984).

These studies indicate the need for a better understanding of the relationship of absorption, distribution, metabolism, and binding of carcinogens in the initiation stage in order to clarify interpretation of the tumorigenic capacity of a chemical. One objective of this study was to determine the effect of different routes of administration and mouse strains on the distribution and binding of urethane to DNA, RNA, and protein in target organs, particularly the skin.

A second objective of this study was to develop a better understanding of the relationship between metabolism of urethane and its own binding to DNA. Lawson and Pound (1971, 1973) found no radioactivity associated with RNA after the administration of (1-^{14}C)-, (2-^{14}C)-, and (carbonyl-

^{14}C)-labeled ethyl carbamate. Upon acid hydrolysis of the DNA nucleotides from the livers of mice treated with [ethyl-1-^{14}C]ethyl carbamate, [^{14}C]ethanol was dissociated and no evidence of binding to the purine and pyrimidine bases or sugars was observed. This led to the conclusion that a phosphate ester in the DNA chain was possibly formed (Pound et al., 1976). The hypothesis was later challenged by Floot et al. (1978), who demonstrated that alkaline sucrose gradients of DNA from ethyl carbamate-treated mice showed no appreciable difference in sedimentation pattern from control mouse liver DNA. More recently, the 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine adducts have been identified in mouse liver RNA after the administration of [ethyl-1-^{14}C]ethyl carbamate (Ribovich et al., 1982). The finding of these adducts suggests that an electrophilic metabolite such as vinyl carbamate epoxide may be the ultimate carcinogen formed in vivo, although the metabolite's existence has not been documented (Dahl et al., 1978). In the second part of this study, [ethyl-1-^{14}C]ethyl carbamate was administered to SENCAR mice in an attempt to determine the nature of the DNA interaction products formed.

METHODS

Animals

Male SENCAR and BALB/c mice (18–31 g) obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) were used in all experiments. All animals were housed in environmentally controlled (22–24°C) rooms for a minimum of 7 d prior to experimentation. Drinking water and food (Lab Blox, Continental Grain Co., Chicago, Ill.) were available ad libitum. The lights were on a 12:12 light–dark cycle (L:0800–2000, D:2000–0800).

Distribution Studies with Urethane

Male SENCAR and BALB/c mice were shaved on the back (area approximately 2.5 X 3.5 cm) and deprived of food, but not water, for the 24-h period prior to dosing and maintained in individual steel cages with wire-mesh floors throughout the experiment in order to minimize oral intake from cross-grooming. Mice were given 2.5 μCi/25 g body weight [ethyl-1-^{14}C]ethyl carbamate at a dose of 0.10 mg/g in double-distilled water or were treated dermally with the same dose applied in acetone. Food was reintroduced 2 h after treatment. The animals were sacrificed by cervical dislocation 1, 6, 12, 24, and 48 h after treatment. The stomach was carefully washed free of nonabsorbed material. The stomach, liver, lungs, and a section of skin from the back (2.5 X 3.5 cm, weighing between 0.75 and 1.0 g) were removed. Portions of the liver, stomach, lungs, or the skin section were minced and digested in 1.0 ml NCS tissue solubilizer (Amersham, Arlington Heights, Ill.) for 24 h at 50°C. After dissolution of

the tissue, 10 ml Aquasol II (New England Nuclear, Boston, Mass.) were added, and the samples were counted on a Packard Tri-Carb scintillation spectrometer (Packard Instrument Company, Inc., Downers Grove, Ill.) for 10 min. [^{14}C]Toluene was then added as an internal standard and the samples were recounted. Since total ^{14}C was counted, results are expressed as urethane equivalents.

Binding Studies with Urethane

Fifty μCi/20 g body weight of [ethyl-1-^{14}C]ethyl carbamate was administered orally at a dose of 0.062 mg/g body weight in double-distilled water or applied dermally in acetone to the shaved backs of the mice. Whole organs of liver, lungs, stomach, and the treated area of skin were excised at either 6 or 48 h after urethane administration and frozen for assaying ^{14}C material bound to DNA, RNA, and protein. These macromolecules were extracted and purified by the Kirby procedure as modified by Diamond et al. (1967). The whole organs were homogenized in 20 volumes of aqueous solution (1% NaCl, 1% triisopropylnaphthalene sulfonic acid, 6% *sec*-butyl alcohol, and 6% *p*-aminosalicylate) using a Potter-Elvehjem homogenizer with a Teflon or glass pestle. The skin samples were frozen in liquid nitrogen and shattered prior to homogenization. One volume of 24:1 chlorofrom/isoamyl alcohol was added, and the two phases were shaken in an ice bath for 10 min. The samples were centrifuged at 10,000 rpm ($\sim$ 10,000 $\times$ g) for 30 min in a Sorvall RC2-B centrifuge (Du Pont Company, Inc., Newton, Conn.) The lipids were removed by the chloroform and the protein separated at the interface of the two layers. The top aqueous layer was removed, washed again in 0.5 volume of chloroform/isoamyl alcohol solution and shaken for 10 min. The DNA was precipitated by the addition of 1 volume of 2-ethoxyethanol to the aqueous layer. RNA was then precipitated after the addition of 2 volumes of ethanol to the remaining aqueous layer and cooling of the samples at -20°C overnight.

The extracted DNA and RNA were washed with 100% ethanol 5 times and diethyl ether twice and then dried under nitrogen. The nucleic acids were incubated in 1.0 ml of 0.5 *M* NaOH and 1.0 ml of 0.01 *M* phosphate buffer. An 0.2-ml aliquot was diluted to 10 ml with phosphate buffer, and the absorbance was measured at 280, 260, and 230 nm in a Beckman ACTA C-III spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). A 260/280 ratio of at least 1.6 was obtained from all samples. Quantitation of DNA and RNA was based on experimentally determined optical density of 251.2 and 210.0, respectively, for a 1% solution of the nucleic acids at pH 7. Approximately 50 mg of the protein was weighed out and solubilized in 2.0 ml of 0.5 *M* NaOH. A 0.2-ml aliquot of this sample was used for protein determination by the Lowry method (1951).

The remaining portion (1.8 ml) was incubated with 1.0 ml NCS tissue solubilizer, and 10 ml Aquasol II was added. Samples were counted on a Packard Tri-Carb scintillation spectrometer (Packard Instrument Company, Inc., Downers Grove, Ill.) for 20 min. [^{14}C]Toluene was then added as an internal standard and the samples were recounted.

Blanks were prepared for all tissues in the same manner and subtracted from the total. Since total ^{14}C was counted, results are expressed as picomoles of urethane equivalents per milligram of macromolecule. Student's *t*-test was used to compare differences between strains for each of the macromolecules. A level of significance of $p = 0.05$ was chosen for these comparisons.

To examine the type of binding of the [^{14}C]urethane, isolated hepatic DNA from SENCAR mice treated orally with the urethane and sacrificed 48 h later was dissolved in 0.1 *M* Tris buffer, pH 7.0, and 0.01 *M* $MgCl_2$ and further purified of any residual RNA or protein by the addition of ribonuclease type IIA (5–75 Kunitz units) and incubation for 20 min. After incubation, proteinase K type XI (5–10 units) was added and again incubated for 10 min at 37°C. The enzymes were twice extracted from the DNA with chloroform/isoamyl alcohol (24:1) by shaking each time for 10 min followed by centrifugation at 2000 rpm for 10 min. The DNA was precipitated from the aqueous layer by the addition of 0.1 volume of 20% NaCl solution and 2 volumes of ethanol and cooled to −20°C for 30 min. The DNA precipitate was then dissolved in 0.01 *M* Tris buffer (pH 7.0) and 0.01 *M* $MgCl_2$. For each milligram of DNA, 260 Kunitz units of deoxyribonuclease 1 from bovine pancreas were added, and the mixture was incubated at 37°C for 6 h. After incubation, an equal volume of 0.1 *M* Tris buffer (pH 9) was added to attain a final pH of approximately 9, and 0.1 units phosphodiesterase type VII from *Crotalus atrox* were added. After incubation for 24 h, another 0.1 units phosphodiesterase were added and incubated an additional 24 h. For studies with deoxyribonucleosides, 2.5 units alkaline phosphatase from *Escherichia coli* type III were added and the samples were incubated at 37°C for an additional 24 h.

Separation of the deoxyribonucleotides was achieved by thin-layer chromatography using 20-cm cellulose plates (Eastman) with fluorescent indicator and a solvent system consisting of *n*-butanol, ethanol, and 5 *N* HCl (3:2:2 by volume). Deoxyribonucleosides were chromatographed on silica-gel 60 thin-layer plates with indicator and a solvent system of concentrated NH_4OH, water, and *n*-butanol (3:5:42 by volume). Authentic 2′-deoxyribonucleosides and 2′-deoxyribonucleotide 5′-monophosphate standards were chromatographed at the same time, and ultraviolet spectra were obtained to verify all bands. The bands were eluted with 1.0 ml double-distilled water. Ten ml Aquasol II (New England Nuclear) was added, and the samples were counted in a Packard Tri-Carb Scintillation Spectrometer.

RESULTS

[Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g) was applied dermally or administered orally to groups of BALB/c or SENCAR mice. After dermal application, the rate of decrease in the amount of labeled urethane in the skin was similar in the BALB/c and SENCAR mouse strains (Fig. 1). However, the label in the skin on a per gram basis after 1 h was approximately 4300 nmol/g in the BALB/c strain, which is insensitive to the tumorgenic effect of urethane, compared to 2200 nmol/g in the sensitive SENCAR mice. The two strains showed significant differences in the amount of label in the skin at all time points except 12 h. The SENCAR mice, however, attained higher concentrations of the [^{14}C]-urethane equivalents at 6 h in the liver, and thereafter in lung and stomach (Figs. 2, 3, and 4). This indicates that urethane is being absorbed more rapidly through the skin to attain higher levels in the other organs. In the BALB/c mice, ^{14}C in the stomach, liver and lungs appeared to level off at 12 h at between 30 and 40 nmol/g tissue. The fairly uniform distribution is in agreement with the early studies of Boyland and Rhoden (1949). In SENCAR mice, the levels of [^{14}C]urethane were significantly higher than in the BALB/c mice, even after 48 h.

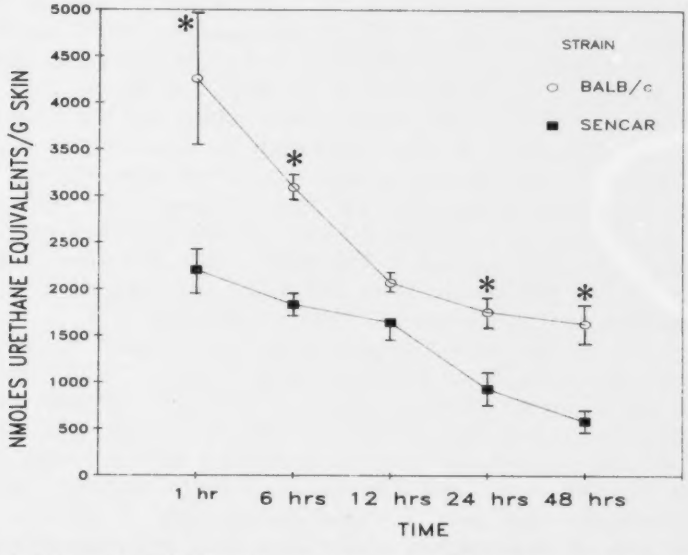

FIGURE 1. Distribution of [^{14}C]urethane in skin when applied dermally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in acetone) was applied dermally to the backs of male BALB/c and SENCAR mice (n = 4–6). The skins were excised at either 1, 6, 12, 24, or 48 h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's t-test.

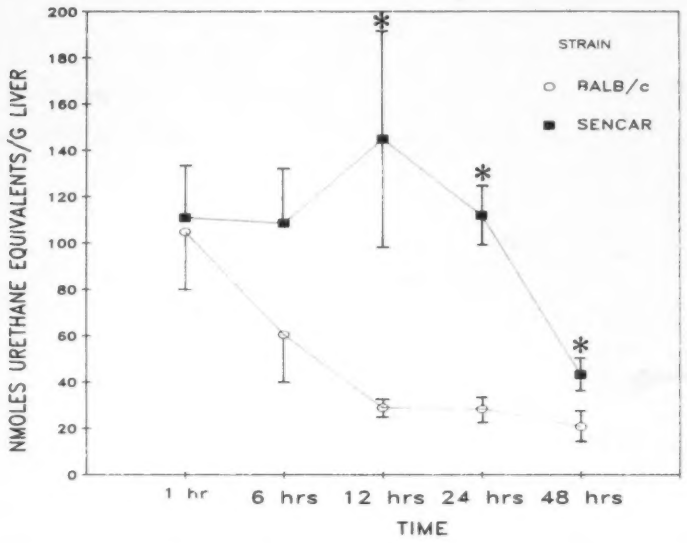

FIGURE 2. Distribution of [^{14}C]urethane in liver when applied dermally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in acetone) was applied dermally to the backs of male BALB/c and SENCAR mice (n = 4–6). The livers were excised at either 1, 6, 12, 24, or 48 h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's t-test.

When tissues were examined from both strains of mice 1 h after oral administration, the concentration of urethane in the stomach tissue was greater in the SENCAR mice than in the BALB/c animals (Fig. 5). This difference was reversed at 6 h but was minimal, and there were no significant differences at 24 h. The differences indicate that urethane is being absorbed more rapidly from the stomach of SENCAR mice, since this is not a result of unabsorbed material, because the stomach was washed of its contents and urethane is known to be rapidly absorbed (Boyland and Rhoden, 1949). The liver, lungs, and skin showed very similar patterns, i.e., the SENCAR mice had significantly higher levels of [^{14}C]urethane 1 h after administration but not at 6 h (Figs. 6, 7 and 8). When the two routes of administration are compared, the concentrations of ^{14}C label in stomach, liver, and lungs after oral administration are very similar and are consistently severalfold higher than when urethane is applied dermally. The concentration in the skin, however, is much lower after oral administration.

At 6 h after the oral administration, there were no significant differences between the two strains in any of the tissues in binding of [^{14}C]-urethane to DNA, RNA, or protein (Fig. 9). The DNA and RNA showed

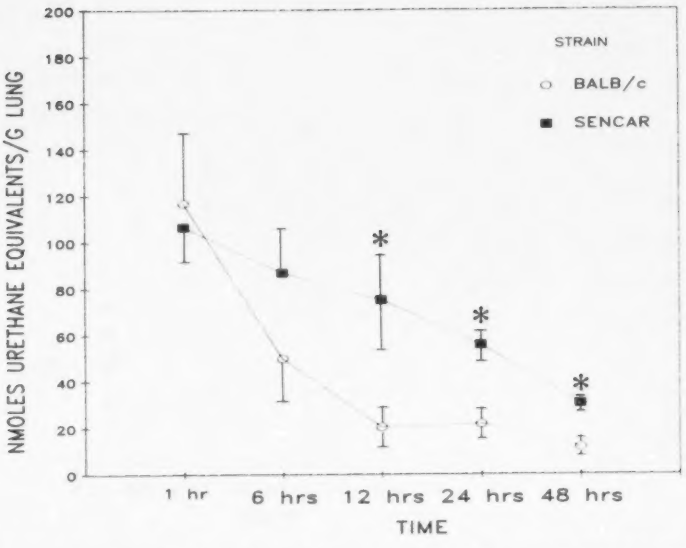

FIGURE 3. Distribution of [^{14}C]urethane in lung when applied dermally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in acetone) was applied dermally to the backs of male BALB/c and SENCAR mice ($n = 4$–6). The lungs were excised at either 1, 6, 12, 24, or 48 h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's t-test.

the same pattern of binding with the liver > stomach > skin > lung. Binding to RNA on a per milligram basis was about $1\frac{1}{2}$ times greater than to DNA. The binding to protein, however, was greater than to either DNA or RNA in the stomach and lung with comparable binding in the liver and skin.

After 48 h there were several significant strain-specific differences in the binding of urethane (Fig. 10). SENCAR mice contained significantly more ^{14}C label bound to the stomach, lung, and skin DNA. The apparent increase in the amount of urethane bound to SENCAR liver DNA was not significant ($p > 0.05$) when compared to BALB/c liver DNA. The binding of urethane to RNA at the same time point indicated that the only tissue of SENCAR mice that bound significantly more [^{14}C]urethane was the stomach. The liver, at 48 h, still maintained the highest level of binding to both DNA and RNA. Binding to protein was significantly greater in the liver and stomach of SENCAR compared to BALB/c mice. The binding of ^{14}C to lung and skin protein was not different between strains.

When comparing the binding of [^{14}C]urethane 6 and 48 h after oral administration, the level of binding decreased over time. The binding to DNA in the liver of BALB/c mice at 6 h was 543 pmol/mg (Fig. 9), while at 48 h it had decreased to 241 pmol (Fig. 10). The stomach also showed

very evident decreases in ^{14}C activity. In BALB/c mice, binding of DNA decreased from 286 to 44 pmol/mg DNA and from 455 to 57 pmol/mg RNA. The dramatic decreases in activity were not as obvious in the lung and skin (Figs. 9 and 10). The skin showed a decline in binding to about 25% of the 6-h level in DNA and 15–20% in RNA.

At 6 h after dermal application, the liver was the only tissue in which significantly more label was bound to DNA in SENCAR mice compared to BALB/c mice (Fig. 11). RNA in the liver showed the same trend, although the difference between the strains was not statistically significant ($p > 0.05$). The binding to DNA and RNA was greatest in the liver > stomach = skin > lung. The binding of urethane to protein was significantly greater in the liver and lungs of SENCAR mice, but the skin from BALB/c mice contained more protein-bound material.

After 48 h, the skin DNA, RNA and protein from BALB/c mice contained significantly more ^{14}C label than did those macromolecules from SENCAR mice (Fig. 12). The liver DNA from SENCAR mice also contained more ^{14}C bound than that of the BALB/c mice. Due to differences in the relative decreases in binding among the tissues between 6 and 48 h, the relative binding to the tissues changed after dermal application of

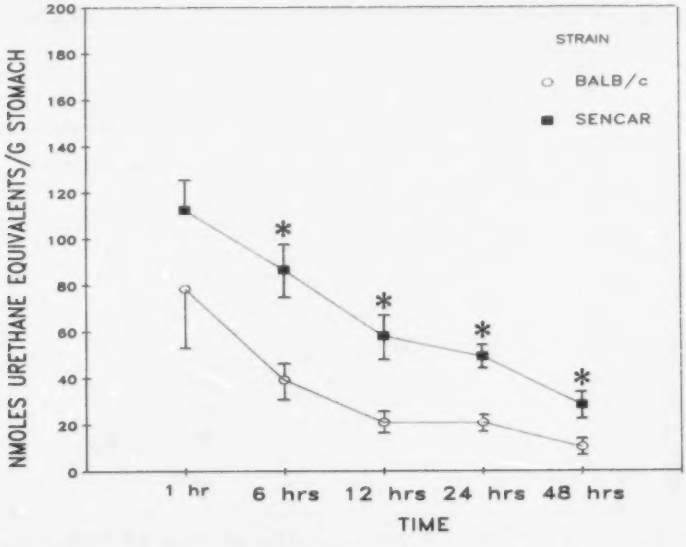

FIGURE 4. Distribution of [^{14}C]urethane in stomach when applied dermally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in acetone) was applied dermally to the backs of male BALB/c and SENCAR mice ($n = 4$–6). The stomachs were excised at either 1, 6, 12, 24, or 48 h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's *t*-test.

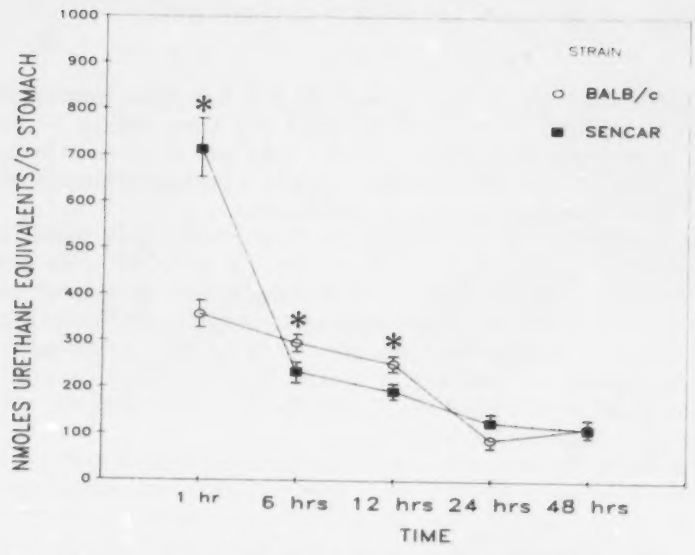

FIGURE 5. Distribution of [^{14}C]urethane in stomach when administered orally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in water) was administered orally to male BALB/c and SENCAR mice ($n = 5$). The stomachs were excised at either 1, 6, 12, 24, or 48-h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's t-test.

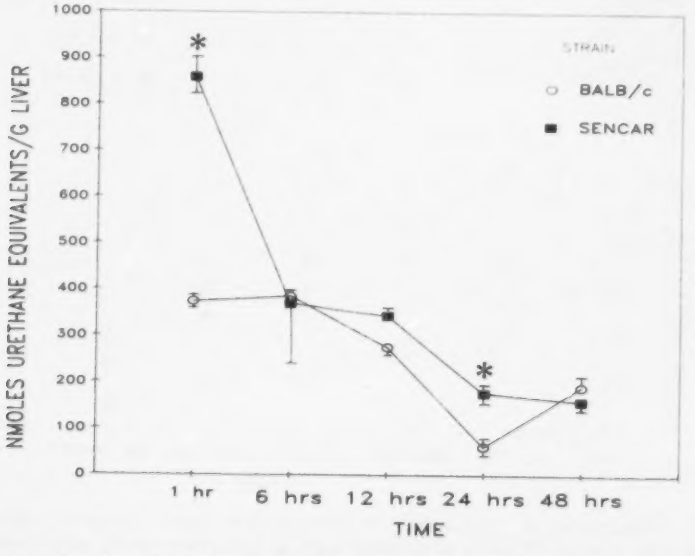

FIGURE 6. Distribution of [^{14}C]urethane in liver when administered orally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in water) was administered orally to male BALB/c and SENCAR mice ($n = 5$). The livers were excised at either 1, 6, 12, 24, or 48 h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's t-test.

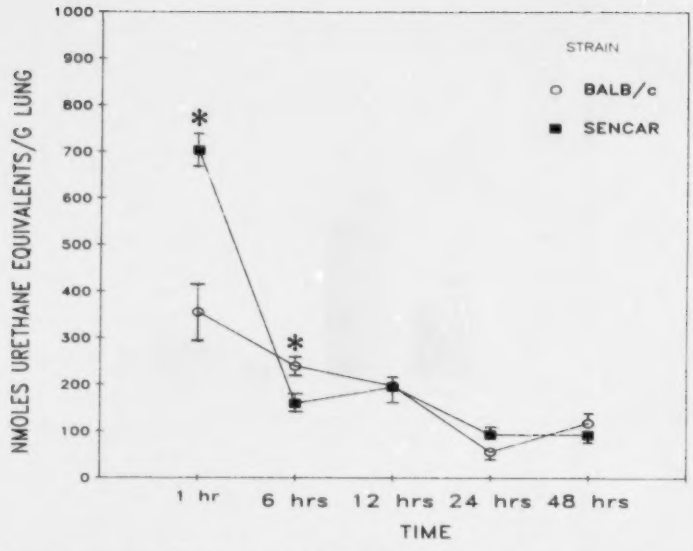

FIGURE 7. Distribution of [^{14}C]urethane in lungs when administered orally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in water) was administered orally to male BALB/c and SENCAR mice ($n = 5$). The lungs were excised at either 1, 6, 12, 24, or 48 h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's t-test.

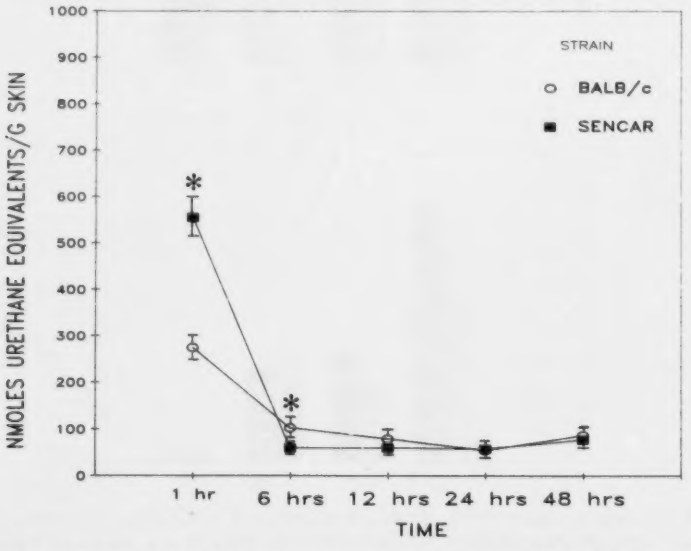

FIGURE 8. Distribution of [^{14}C]urethane in skin when administered orally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (2.5 μCi/25 g at a dose of 0.10 mg/g in water) was administered orally to male BALB/c and SENCAR mice ($n = 5$). The skins were excised at either 1, 6, 12, 24, or 48 h after treatment, and total ^{14}C label was counted. Value represents mean ± SE. Asterisks indicate differences between strains at the same time point ($p < 0.05$) using Student's t-test.

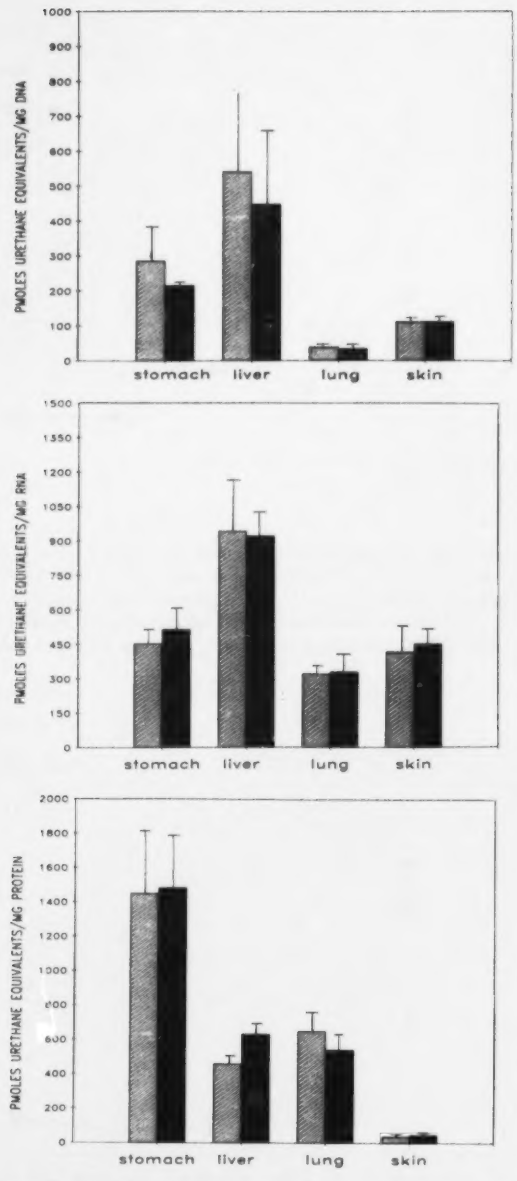

FIGURE 9. Covalent binding to DNA, RNA, and protein of [^{14}C]urethane administered orally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (50 μCi/20 g body weight at a dose of 0.062 mg/g body weight in water) was administered orally to shaved male BALB/c (hatched bar) and SENCAR (solid bar) mice, $n = 4$–6. The tissues were excised at 6 h, and DNA, RNA, and protein were isolated. Total ^{14}C label covalently bound was counted as described in methods section. Value represents mean ± SE.

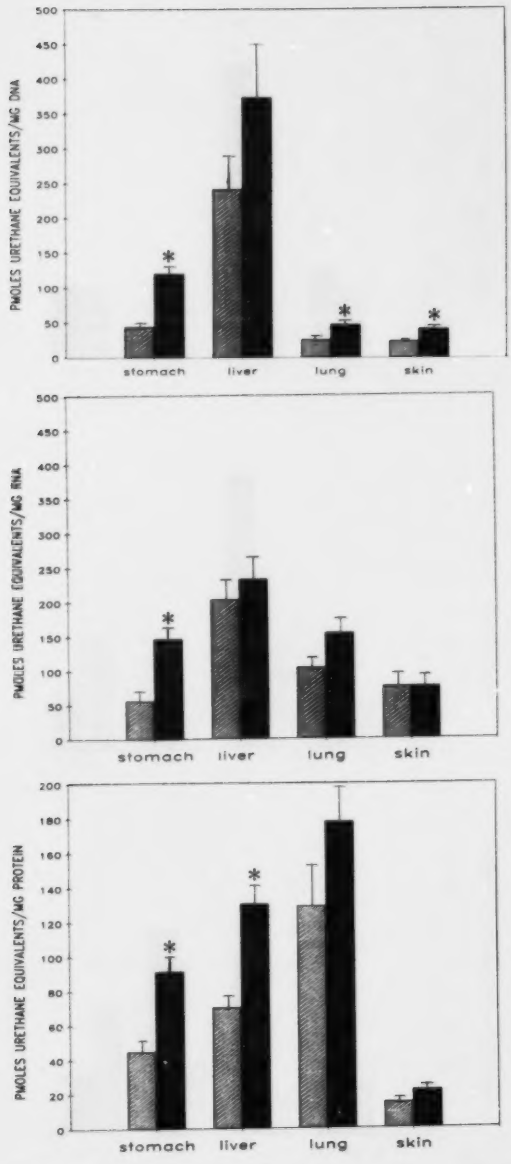

FIGURE 10. Covalent binding to DNA, RNA, and protein of [^{14}C]urethane adminstered orally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (50 μCi/20 g body weight at a dose of 0.062 mg/g body weight in water) was administered orally to shaved male BALB/c (hatched bar) and SENCAR (solid bar) mice, $n = 4$–6. The tissues were excised at 48 h, and DNA, RNA, and protein were isolated. Total ^{14}C label covalently bound was counted as described in methods section. Value represents mean ± SE. Asterisks indicate differences between strains in the same tissue ($p < 0.05$).

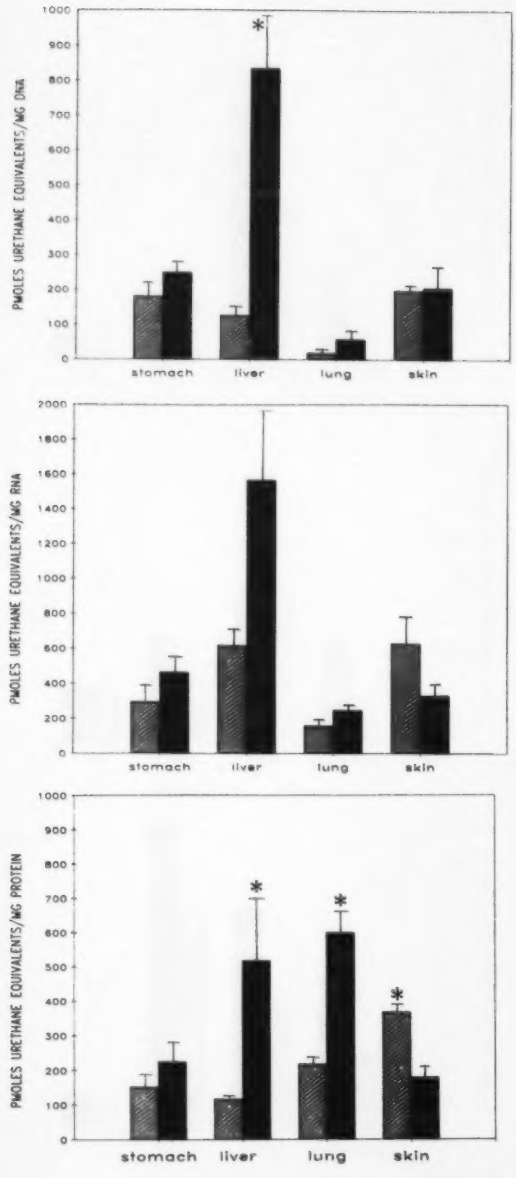

FIGURE 11. Covalent binding to DNA, RNA, and protein of [^{14}C]urethane applied dermally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (50 μCi/20 g body weight at a dose of 0.062 mg/g body weight in acetone) was applied dermally to the backs of shaved male BALB/c (hatched bar) and SENCAR mice (solid bar), $n = 4$–6. The tissues were excised at 6 h, and DNA, RNA, and protein were isolated. Total ^{14}C label covalently bound was counted as described in methods section. Value represents mean ± SE. Asterisks indicate differences between strains in the same tissue ($p < 0.05$).

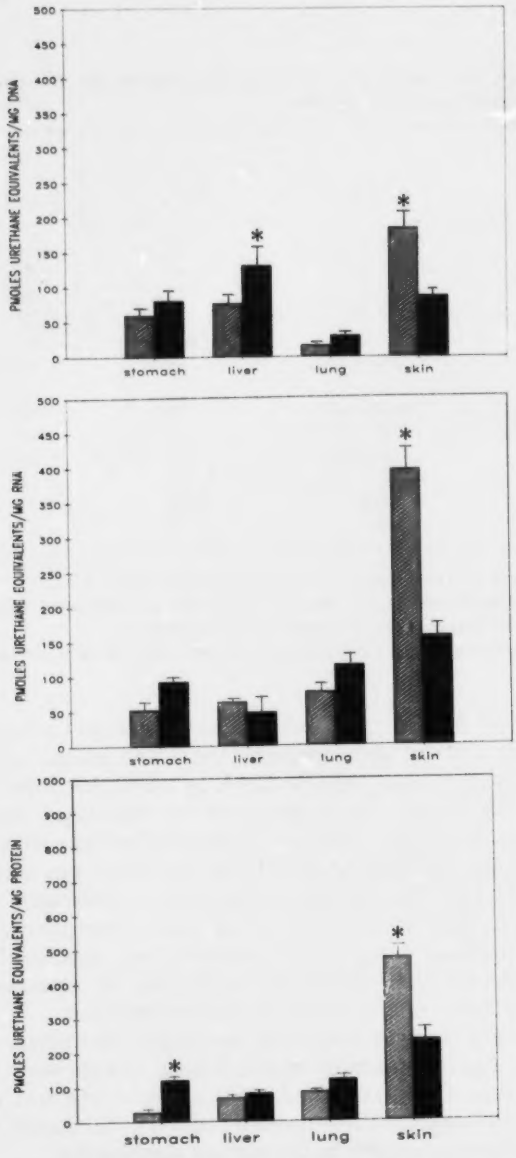

FIGURE 12. Covalent binding to DNA, RNA, and protein of [^{14}C]urethane applied dermally to BALB/c and SENCAR mice. [Ethyl-1-^{14}C]ethyl carbamate (50 μCi/20 g body weight at a dose of 0.062 mg/g body weight in acetone) was applied dermally to the backs of shaved male BALB/c (hatched bar) and SENCAR mice (solid bar), $n = 4$–6. The tissues were excised at 48 h, and DNA, RNA, and protein were isolated. Total ^{14}C label covalently bound was counted as described in methods section. Value represents mean ± SE. Asterisks indicate differences between strains in the same tissue ($p < 0.05$).

TABLE 1. Thin-Layer Chromatography of Deoxyribonucleotides and Deoxyribonucleosides from Liver of [^{14}C] Urethane-Treated SENCAR Mice

Elution band	R_f range[a]	cpm		
		Exp. 1	Exp. 2	Exp. 3[b]
dGMP	0.226–0.245	38	37	28
dAMP	0.361–0.381	47	45	31
dCMP	0.707–0.728	23	25	22
dTMP	0.930–0.932	20	21	17
Origin	0.000	835	465	360
Background control	0.000	18	17	19
d-Guanosine	0.164–0.168	25	20	25
d-Cytidine	0.228–0.277	23	19	21
Thymidine	0.406–0.411	22	25	22
d-Adenosine	0.506–0.600	25	18	22
Origin	0.000	1053	514	936
Background control	0.000	27	17	23

[a]Cellulose plates and a solvent system of *n*-butanol, ethanol, and 5 *N* HCl (3:2:2 by volume) were used for the deoxyribonucleotides and silica-gel plates with concentranted NH_4OH, water, and *n*-butanol (3:5:42 by volume) were used for the deoxyribonucleosides.

[b]Deoxyribonucleotides and deoxyribonucleosides are not from the same mouse in this experiment.

urethane. After 48 h, skin appeared to bind the greatest amount of urethane equivalents, followed by the stomach, liver, and lungs, all being similar.

When nucleotides from hepatic DNA of SENCAR mice treated orally and sacrificed 48 h later were separated by thin-layer chromatography, almost all the radioactivity from the [ethyl-^{14}C] ethyl carbamate remained at the origin of the cellulose plates (Table 1). There was little or no incorporation of label into the normal nucleotides, as indicated by the lack of increase over background of those spots that corresponded to authentic standard deoxyribonucleotides. In addition, no unidentified bands of material were found that would be indicative of modified nucleotides migrating at a different rate from the normal nucleotides.

The experiment was repeated with enzymatic hydrolysis of the nucleotides to their corresponding nucleosides. Similar results were found, in that most of the radioactivity remained at the origin and was not associated with the normal deoxyribonucleosides (Table 1). No bands were observed other than those corresponding to the normal nucleosides.

DISCUSSION

In the initiation–promotion assay, differences in the tumor incidence due to route of administration and strain of mouse have complicated the use of this assay for the prediction of risk (Dahl et al., 1978; Bull et al.,

1981, 1983; Hennings et al., 1981). The first objective of this study was to determine if there is a difference in the distribution of urethane. When levels of [^{14}C]urethane in the skin, lungs, liver, and stomach tissues were examined 1 h after oral administration, the concentration of urethane in the stomach was greater in the SENCAR mice than in the BALB/c mice. This difference was not generally observed at 6, 12, 24, and 48 h, indicating that it was probably the result of a greater absorption in the SENCAR mice. A similar finding was observed in the liver, lungs, and skin. On the other hand, it could be that the BALB/c mice absorbed the material faster within the first hour and little remained in the stomach tissue. Since there was little difference in concentration of urethane in these tissues between the strains following oral administration, it would appear that absorption and distribution of urethane do not account for the strain differences seen in the papilloma studies (Robinson et al., 1984).

When [^{14}C]urethane was applied dermally, the amount of label on a per gram of skin basis was greater in the insensitive BALB/c strain than in the sensitive SENCAR mice throughout the period of testing. It appears that the absorption of the [^{14}C]urethane is retarded in the BALB/c mice compared to the SENCAR strain. This could explain why the SENCAR mice showed higher levels of labeled material in the liver, lung, and stomach than did the BALB/c mice. The differences in these organs then is in agreement with the sensitivities between the two mouse strains, except in the target organ of primary interest, the skin.

A second objective was to determine if the macromolecular binding could account for the route and strain differences in tumor induction following urethane administration. It may be possible for different tissues to bind different percentages of a compound upon exposure to a given concentration. When [^{14}C]urethane was administered orally, there were no significant differences in binding to DNA, RNA or protein between the two strains 6 h after administration. That binding of the ethyl moiety of the [^{14}C]ethyl carbamate was occurring rather than simply incorporation of labeled ethanol liberated from urethane metabolism was demonstrated by the lack of incorporation of label into the normal nucleotides or nucleosides isolated from hepatic DNA. Comparing the four tissues examined, binding to DNA and RNA was highest in the liver, possibly because of the greater drug-metabolizing ability of this tissue, which would result in more activation to reactive intermediates. Also, following absorption, much of the material would be presented directly to the liver prior to systemic distribution, i.e., the first-pass effect might be important.

Although there were no differences between the 2 strains after 6 h, 48 h after oral administration of the urethane, differences were apparent. In the stomach, lung, and skin, binding was significantly greater in the SENCAR mice than in the BALB/c mice. Binding to DNA in the BALB/c mice was between 36 and 65% that of SENCAR mice DNA, depending on the tissue examined. It appears that by 48 h significant repair has

occurred in the animals, and the SENCAR mice retained higher levels of ^{14}C-macromolecular binding. The decreases in binding to RNA and protein indicate that turnover has also occurred during this time period.

At 6 h after the dermal application of [^{14}C]urethane, SENCAR mouse liver DNA had approximately seven times more ^{14}C bound than did that of the BALB/c mouse, even though the distribution studies demonstrated no significant difference between the two strains. This suggests that the liver of this strain has a greater ability to activate the parent compound to an intermediate that can bind to DNA. The other tissues did not show a strain-specific difference with respect to DNA binding at this time.

When comparing the two routes of administration, the values were similar except for the liver as already mentioned and the skin. Although the concentration of urethane was much higher 6 h after dermal application than after oral administration, the binding to DNA was only slightly greater, indicating that the amount of urethane presented to the tissue is probably not the limiting factor in determining the degree of macromolecular binding.

Binding to macromolecules 48 h after dermal application was similar between strains except in skin, where binding to DNA, RNA, and protein was significantly higher in the skin of BALB/c mice. This may be due to the longer retention and higher levels of [^{14}C]urethane in the skin of BALB/c mice observed in the distribution studies. This would allow more time for urethane to react with macromolecules. Binding to skin DNA was increased twofold in SENCAR mice to 87 pmol/mg DNA when applied dermally compared to when it was administered orally, while binding in BALB/c mice was increased about 7 times (184 pmol/mg DNA). This indicates that BALB/c mice can bind significantly more ^{14}C label, yet the papilloma studies by Bull et al. (1983) indicate that BALB/c mice are more resistant to urethane-initiated papilloma formation. The finding that the SENCAR mice have lower levels of DNA binding in skin than do BALB/c mice suggests that qualitative differences in the types of DNA adduct formed or repair of these adducts may be involved in the different susceptibilities of these two strains, or that it could be a matter of expression of the damage during the promotional phase.

The radioactivity associated with the administration of [ethyl-1-^{14}C]-ethyl carbamate to SENCAR mice remained in a fraction of the liver DNA that did not chromatograph with the normal bases or would be characteristic of a slightly modified base, such as reported by Ribovich et al. (1982) for isolated RNA. One possible explanation for this finding is that dinucleotides are formed upon the administration of ethyl carbamate that are resistant to cleavage of the 3′,5′-phosphate linkage after digestion with snake venom phosphodiesterase. This would explain the chromatographic elution characteristics of the ethyl carbamate adducts. The most likely explanation for this would be that ethyl carbamate alkylated the phosphate to form a

phosphotriester that prevented the phosphodiesterase from hydrolyzing the ester.

The concept of phosphate alkylation by urethane was speculated by Pound et al. (1976). Floot et al. (1978) later showed that DNA fragmentation as a result of alkali-labile phosphotriester formation did not occur in ethyl carbamate treated mice. The analysis of samples of [^{3}H]thymidine-labeled liver DNA subjected to alkaline gradient centrifugation showed that mice treated with urethane only had one labeled band of DNA, whereas animals treated with diethylnitrosamine showed multiple bands of fragmented DNA. The assay requires, however, that at least 2000 DNA breaks/cell must occur before DNA profiles from treated and untreated animals can be distinguished. It is very possible that the assay is not sensitive enough for identifying phosphotriester formation from ethyl carbamate-induced alkylation.

REFERENCES

Berenblum, I. and Haran-Ghera, H. 1957. Papilloma formation in the forestomach of the mouse following oral administration of urethane (ethyl carbamate). *Cancer Res.* 17:329–331.

Boyland, E. and Rhoden, E. 1949. The distribution of urethane in animal tissues as determined by a micro-diffusion method, and the effect of urethane treatment on enzymes. *Biochem. J.* 44: 528–531.

Bull, R. J., Robinson, M., Glass, J., Cmehil, D., and Orthoefer, J. 1981. Induction of skin papillomas in the SENCAR mouse as a tier 2 carcinogenesis bioassay. *Toxicologist* 1:129.

Bull, R. J., Robinson, M., and Laurie, D. 1983. Combined mouse lung adenoma and skin initiation/promotion assay for chemical carcinogens. *Toxicologist* 3:145;

Bull, R. J., Robinson, M., Laurie, R. D., Stoner, G. D., Greisinger, E., Meier, J. R., and Stober, J. 1984. Carcinogenic effects of acrylamide in SENCAR and A/J mice. *Cancer Res.* 44:107–111.

Dahl, G. A., Miller, J. A., and Miller, E. C. 1978. Vinyl carbamate as a promutagen and a more carcinogenic analog of ethyl carbamate. *Cancer Res.* 38:3793–3804.

Diamond, L., Defendi, V. and Brooks, P. 1967. The interaction of 7,12-dimethylbenz(a)anthracene with cells sensitive and resistant to toxicity induced by this carcinogen. *Cancer Res.* 27:890–897.

DiGiovanni, J., Slaga, T. J., and Boutwell, R. K. 1980. Comparison of the tumor-initiating activity of 7,12-dimethylbenz(a)anthracene and benzo(a)pyrene in female SENCAR and CD-1 mice. *Carcinogenesis* 1:381–389.

Floot, B. G. J., Philippus, E. J., Scherer, E., and Den Engelse, L. 1978. Effect of urethane administration on the sedimentation patterns of mouse and rat liver DNA in alkaline sucrose gradients. *Chem.-Biol. Interact.* 21:331–336.

Hennings, H., Devor, D., Wenk, M. L., Slaga, T. J., Former, B., Colburn, N. H., Bowden, G. T., Elgjo, K., and Yuspa, S. H. 1981. Comparison of two-stage epidermal carcinogenesis initiated by 7,12-dimethylbenz(a)anthracene or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in newborn and adult SENCAR and BALB/c mice. *Cancer Res.* 41:773–779.

Heston, W. E., Vlahakis, G., and Deringer, M. K. 1960. High incidence of spontaneous hepatomas and the increase of this incidence with urethane in C3H, C3Hf, and C3He male mice. *J. Natl. Cancer Inst.* 24:425–435.

Klein, M. 1966. Influence of age on induction of hepatomas and other tumors in infant mice. *J. Natl. Cancer Inst.* 36:1111–1120.

Lawson, T. A. and Pound, A. W. 1971. The reaction of urethane with mouse liver nucleic acids *in vivo*. *Pathology* 3:223–225.

Lawson, T. A. and Pound, A. W. 1973. The interaction of carbon-14-labelled alkyl carbamates labelled in the alkyl and carbonyl positions, with DNA *in vivo*. *Chem. Biol Interact.* 6:99–105.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–271.

Nettleship, A., Henshaw, P., and Meyer, H. 1943. Induction of pulmonary tumors in mice with ethyl carbamate (urethane). *J. Natl. Cancer Inst.* 4:309–319.

Pound, A. W. and Bell, J. R. 1962. The influence of croton oil stimulation on tumour initiation by urethane in mice. *Br. J. Cancer* 16:690–695.

Pound, A. W., Franke, F., and Lawson, T. A. 1976. The binding of ethyl carbamate to DNA of mouse liver *in vivo:* The nature of the bound molecule and the site of binding. *Chem.-Biol. Interact.* 14:149–163.

Ribovich, M. L., Miller, J. A., Miller, E. C., and Timmins, L. G. 1982. Labeled 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine in hepatic RNA of mice given (ethyl-1,2-^{3}H or ethyl-1-^{14}C) ethyl carbamate (urethan). *Carcinogenesis* 3:539–546.

Robinson, M., Bull, R. J., Knutsen, G., and Shields, R. P. 1984. The SENCAR mouse: Its use in both the lung adenoma and skin initiation/promotion assays. *Toxicologist* 4:42.

Salaman, M. H. and Roe, F. J. C. 1953. Incomplete carcinogens: Ethyl carbamate (urethane) as an initiator of skin tumour formation in the mouse. *Br. J. Cancer* 7:472–481.

Slaga, T. J., Fischer, S. M., Weeks, C. E., Klein-Szanto, A. S. P., and Reiners, J. 1982. Studies on the mechanism involved in multistage carcinogenesis in mouse skin. *J. Cell. Biochem.* 18:99–119.

Yamamoto, R. S., Weisburger, J. H., and Weisburger, E. K. 1971. Controlling factors in urethane carcinogenesis in mice: Effect of enzyme inducers and metabolic inhibitors. *Cancer Res.* 31: 483–486.

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